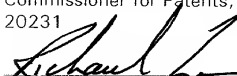


## JOINT INVENTORS

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Richard Zimmermann

## APPLICATION FOR UNITED STATES LETTERS PATENT

# **S P E C I F I C A T I O N**

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TO ALL WHOM IT MAY CONCERN:

Be it known that we, David E. Lowery a citizen of the United States of America, residing at 1207 Woodland Drive, Portage, 49024, in the State of Michigan and Michael J. Kennedy a citizen of the United States of America, residing at 2364 Quincy Avenue, Portage, 49024, in the State of Michigan have invented a new and useful **SALMONELLA VACCINE MATERIALS AND METHODS**, of which the following is a specification.

## SALMONELLA VACCINE MATERIALS AND METHODS

The present invention claims priority of U.S. provisional application no. 60/190,178 filed March 17, 2000, the entire disclosure of which is incorporated by reference herein.

5

### FIELD OF THE INVENTION

The present invention relates generally to genetically engineered salmonellae, which are useful as live vaccines.

### BACKGROUND OF THE INVENTION

10 Diseases caused by *Salmonella* bacteria range from a mild, self-limiting diarrhea to serious gastrointestinal and septicemic disease in humans and animals. *Salmonella* is a gram-negative, rod-shaped, motile bacterium (nonmotile exceptions include *S. gallinarum* and *S. pullorum*) that is non-spore forming. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces, raw meats, raw poultry, and raw seafoods.

15 *Salmonella* infection is a widespread occurrence in animals, especially in poultry and swine, and is one of the most economically damaging of the enteric and septicemic diseases that affect food producing animals. Although many serotypes of *Salmonella* have been isolated from animals, *S. choleraesuis* and *S. typhimurium* are the two most frequently isolated serotypes associated with clinical salmonellosis in

20 pigs. In swine, *S. typhimurium* typically causes an enteric disease, while *S. choleraesuis* (which is host-adapted to swine) is often the etiologic agent of a fatal septicemic disease with little involvement of the intestinal tract. *S. dublin* and *S. typhimurium* are common causes of infection in cattle; of these, *S. dublin* is host adapted to cattle and is often the etiologic agent of a fatal septicemic disease. Other

25 serotypes such as *S. gallinarum* and *S. pullorum* are important etiologic agents of salmonellosis in avian and other species. Although these serotypes primarily infect animals, *S. dublin* and *S. choleraesuis* also often cause human disease.

Various *Salmonella* species have been isolated from the outside of egg shells, including *S. enteritidis* which may even be present inside the egg yolk. It has

been suggested that the presence of the organism in the yolk is due to transmission from the infected layer hen prior to shell deposition. Foods other than eggs have also caused outbreaks of *S. enteritidis* disease in humans.

*S. typhi* and *S. paratyphi* A, B, and C produce typhoid and typhoid-like fever in humans. Although the initial infection with salmonella typically occurs through the gastrointestinal tract, typhoid fever is a systemic disease that spreads throughout the host and can infect multiple organ sites. The fatality rate of typhoid fever can be as high as 10% (compared to less than 1% for most forms of salmonellosis). *S. dublin* has a 15% mortality rate when the organism causes septicemia in the elderly, and *S. enteritidis* has an approximately 3.6% mortality rate in hospital/nursing home outbreaks, with the elderly being particularly affected.

Numerous attempts have been made to protect humans and animals by immunization with a variety of vaccines. Many of the vaccines provide only poor to moderate protection and require large doses to be completely efficacious. Previously used vaccines against salmonellae and other infectious agents have generally fallen into four categories: (i) specific components from the etiologic agent, including cell fractions or lysates, intact antigens, fragments thereof, or synthetic analogs of naturally occurring antigens or epitopes (often referred to as subunit vaccines); (ii) anti-idiotypic antibodies; (iii) the whole killed etiologic agent (often referred to as killed vaccines); or (iv) an avirulent (attenuated) derivative of the etiologic agent used as a live vaccine.

Reports in the literature have shown that attenuated live vaccines are more efficacious than killed vaccines or subunit vaccines for inducing protective immunity. Despite this, high doses of live vaccines are often required for efficacy and few live-attenuated *Salmonella* vaccines are commercially available. Ideally, an effective attenuated live vaccine retains the ability to infect the host without causing serious disease and is also capable of stimulating humoral (antibody-based) immunity and cell-mediated immunity sufficient to provide resistance to any future infection by virulent bacteria.

Several attenuation strategies have been utilized to render *Salmonella* avirulent [Cardenas et al., Clin Microbial Rev. 5:328-342 (1992); Chatfield et al., Vaccine 7:495-498 (1989); Curtiss, in Woodrow et al., eds., New Generation Vaccines, Marcel Dekker, Inc., New York, p. 161 (1990); Curtiss et al., in Kohler et al., eds., Vaccines: new concepts and developments. Proceedings of the 10th Int'l Convocation of Immunology, Longman Scientific and Technical, Harlow, Essex, UK, pp. 261-271 (1987); Curtiss et al., in Blankenship et al., eds., Colonization control of human bacterial enteropathogens in poultry, Academic Press, New York, pp. 169-198 (1991)]. These strategies include the use of temperature sensitive mutants [e.g., Germanier et al., Infect Immun. 4:663-673 (1971)], aromatic and auxotrophic mutants (e.g., -aroA, -asd, -cys, or -thy [Galan et al., Gene 94:29-35 (1990); Hoiseth et al., Nature 291:238-239 (1981); Robertsson et al., Infect Immun. 41:742-750 (1983); Smith et al., Am J Vet Res. 45:59-66 (1984); Smith et al., Am J Vet Res. 45:2231-2235 (1984)]], mutants defective in purine or diaminopimelic acid biosynthesis (e.g.,  $\Delta$ pur and  $\Delta$ ap [Clarke et al., Can J Vet Res. 51:32-38 (1987); McFarland et al., Microb Pathog. 3:129-141 (1987); O'Callaghan et al., Infect Immun. 56:419-423 (1988)]], strains altered in the utilization or synthesis of carbohydrates (e.g.,  $\Delta$ galE [Germanier et al., Infect Immun. 4:663-673 (1971); Hone et al., J Infect Dis. 156:167-174 (1987)]], strains altered in the ability to synthesize lipopolysaccharide (e.g., *galE*, *pmi*, *rfa*) or cured of the virulence plasmid, strains with mutations in one or more virulence genes (e.g., *invA*) and mutants altered in global gene expression (e.g., -cya -crp, ompR or -phoP [Curtiss (1990), *supra*; Curtiss et al. (1987), *supra*; Curtiss et al. (1991), *supra*]).

In addition, random mutagenesis techniques have been used to identify virulence genes expressed during infection in an animal model. For example, using a variety of approaches, random mutagenesis is carried out on bacteria followed by evaluation of the mutants in animal models or tissue culture systems, such as Signature-Tagged Mutagenesis (STM) [see U.S. Patent No. 5,876,931].

However, published reports have shown that attempts to attenuate *Salmonella* by these and other methods have led to varying degrees of success and

demonstrated differences in both virulence and immunogenicity [Chatfield et al., Vaccine 7:495-498 (1989); Clarke et al., Can J Vet Res. 51:32-38 (1987); Curtiss (1990), *supra*; Curtiss et al. (1987), *supra*; Curtiss et al. (1991), *supra*]. Prior attempts to use attenuation methodologies to provide safe and efficacious live vaccines have encountered a number of problems.

First, an attenuated strain of *Salmonella* that exhibits partial or complete reduction in virulence may not retain the ability to induce a protective immune response when given as a vaccine. For instance,  $\Delta$ aroA mutants and galE mutants of *S. typhimurium* lacking UDP-galactose epimerase activity were found to be immunogenic in mice [Germanier et al., Infect Immun. 4:663-673 (1971), Hohmann et al., Infect Immun. 25:27-33 (1979); Hoiseth et al., Nature, 291:238-239 (1981); Hone et al., J. Infect Dis. 156:167-174 (1987)] whereas  $\Delta$ asd,  $\Delta$ thy, and  $\Delta$ pur mutants of *S. typhimurium* were not [Curtiss et al. (1987), *supra*, Nnalue et al., Infect Immun. 55:955-962 (1987)]. All of these strains, nonetheless, were attenuated for mice when given orally or parenterally in doses sufficient to kill mice with the wild-type parent strain. Similarly,  $\Delta$ aroA,  $\Delta$ asd,  $\Delta$ thy, and  $\Delta$ pur mutants of *S. choleraesuis* were avirulent in mice, but only  $\Delta$ aroA mutants were sufficiently avirulent and none were effective as live vaccines [Nnalue et al., Infect Immun. 54:635-640 (1986); Nnalue et al., Infect Immun. 55:955-962 (1987)].

Second, attenuated strains of *S. dublin* carrying mutations in *phoP*, *phoP* *crp*, [*crp*-*cdt*] *cya*, *crp* *cya* were found to be immunogenic in mice but not cattle [Kennedy et al., Abstracts of the 97th General Meeting of the American Society for Microbiology. B-287:78 (1997)]. Likewise, another strain of *S. dublin*, SL5631, with a deletion affecting gene *aroA* was highly protective against lethal challenge to a heterologous challenge strain in mice [Lindberg et al., Infect Immun. 61:1211-1221 (1993)] but not cattle [Smith et al., Am J Vet Res. 54:1249-1255 (1993)].

Third, genetically engineered *Salmonella* strains that contain a mutation in only a single gene may spontaneously mutate and "revert" to the virulent state. The introduction of mutations in two or more genes tends to provide a high level of safety against restoration of pathogenicity by recombination [Tackett et al.,

Infect Immun. 60:536-541 (1992)]. However, the use of double or multiple gene disruptions is unpredictable in its effect on virulence and immunogenicity; the introduction of multiple mutations may overattenuate a bacteria for a particular host [Linde et al., Vaccine 8:278-282 (1990); Zhang et al., Microb. Pathog., 26(3):121-130 (1999)].

Of interest to the present invention is the identification of pathogenicity islands (PAIs) in *Salmonella* and other bacteria, which are large, sometimes unstable, chromosomal regions harboring clusters of genes that often define virulence characteristics in enteric bacteria. The DNA base composition of PAIs often differs from those of the bacterial chromosomes in which they are located, indicating that they have probably been acquired by horizontal gene transfer. One *Salmonella* pathogenicity island containing genes required for epithelial cell invasion has been identified at around 63 centisomes (cs) on the *S. typhimurium* chromosome, and has been shown to contain genes encoding components of a type III (contact-dependent) secretion system, secreted effector proteins, and associated regulatory proteins [Millis et al., *Mol Microbiol* 15(4):749-59 (1995)] A second *Salmonella* PAI of 40 kb is located at 30.7 and has been designated *Salmonella* pathogenicity island 2 (SPI2) [Shea et al., *Proc. Nat'l Acad. Sci. USA*, 19;93(6):2593-2597 (1996)]. Nucleotide sequence analysis of regions of SPI2 revealed genes encoding a second type III secretion apparatus that has been suggested to be involved at a stage of pathogenesis subsequent to epithelial cell penetration. Mutations in some genes within SPI2 have been shown to result in attenuation of bacterial virulence in mice. See U.S. Patent No. 5,876,931; Shea et al., *Proc. Natl. Acad. Sci. USA*, 93:2593-2597 (1996); Ochman et al., *Proc Natl Acad Sci USA*, 93(15):7800-7804 (1996); Hensel et al., *J. Bacteriol.*, 179(4):1105-1111 (1997); Hensel et al., *Molec. Microbiol.*, 24(1):155-167 (1997); Dunyak et al., poster presented at 97th General Meeting of the American Society for Microbiology (1997), p. 76.

A need continues to exist for more safe and efficacious live attenuated *Salmonella* vaccines that ideally do not need to be administered at very large doses.

### BRIEF SUMMARY OF THE INVENTION

The present invention relates to safe and efficacious vaccines employing one or more strains of attenuated mutant gram-negative bacteria in which one or more genes homologous to genes within *Salmonella* pathogenicity island 2 (SPI2) have been inactivated, preferably by deletion of about 5% to about 100% of the gene, most preferably by deletion of about 50% or more of the gene. Specifically contemplated are vaccines comprising one or more species of attenuated mutant *Salmonella* bacteria in which one or more genes, and preferably two or more genes, within SPI2 have been inactivated. In a preferred embodiment, one or more of the *ssa* genes, particularly *ssaT*, *ssaJ*, *ssaC* or *ssaM* genes, have been inactivated in the mutant bacteria. The invention is based on results of extensive safety and efficacy testing of these vaccines, including vaccines containing more than one serotype of *Salmonella*, in animal species other than rodents, including cattle and pigs.

According to one aspect of the present invention, vaccine compositions are provided that comprise an immunologically protective amount, of a first attenuated mutant *Salmonella* bacterium in which one or more *ssa* genes are inactivated. In one embodiment, the *ssa* genes are selected from the group consisting of *ssaT*, *ssaJ*, *ssaC* and *ssaM*. Suitable amounts will vary but may include about  $10^9$  bacteria or less. In these mutant bacteria, the inactivated gene(s) is/are preferably inactivated by deletion of a portion of the coding region of the gene. Alternatively, inactivation is effected by insertional mutation. Any species of *Salmonella* bacteria, particularly *S. enterica* subspecies and subtypes, may be mutated according to the invention, including *Salmonella* from serogroups A, B, C<sub>1</sub>, C<sub>2</sub>, D<sub>1</sub> and E<sub>1</sub>. All of the *Salmonella* serovars belong to two species: *S. bongori* and *S. enterica*. The six subspecies of *S. enterica* are: *S. enterica* subsp. *enterica* (I or 1), *S. enterica* subsp. *salamae* (II or 2), *S. enterica* subsp. *arizonae* (IIIa or 3a), *S. enterica* subsp. *diarizonae* (IIIb or 3b), *S. enterica* subsp. *houtenae* (IV or 4), *S. enterica* subsp. *indica* (VI or 6). Exemplary subspecies include: *S. Choleraesuis*, *S. Typhimurium*, *S. Typhi*, *S. Paratyphi*, *S. Dublin*, *S. Enteritidis*, *S. Gallinarum*, *S. Pullorum*, *Salmonella* *Anatum*, *Salmonella* *Hadar*, *Salmonella* *Hamburg*, *Salmonella* *Kentucky*, *Salmonella*

*Miami, Salmonella Montevideo, Salmonella Ohio, Salmonella Sendai, Salmonella Typhisuis.*

Two or more virulence genes may be inactivated in the mutant *Salmonella* bacteria, of which at least one gene is a gene within SPI2. In one aspect, the gene is an *ssa* gene. Preferably, two genes selected from the group consisting of *ssaT*, *ssaJ* and *ssaC* have been inactivated. Most preferably, the combination of *ssaT* and *ssaC*, or *ssaT* and *ssaJ* have been inactivated.

The vaccine composition may further comprise a second attenuated mutant *Salmonella* bacterium in which one or more *ssa* genes have been inactivated. In one aspect, the *ssa* gene is selected from the group consisting of *ssaT*, *ssaJ* and *ssaC*. Preferably, the first and second mutant *Salmonella* bacteria are of different serotypes. For cattle, vaccines comprising both *S. dublin* and *S. typhimurium* are preferred.

The invention also provides methods of immunizing, i.e., conferring protective immunity on, an animal by administering the vaccine compositions of the invention. Signs of protective immunity are described below. The invention further provides methods of reducing transmission of infection by administering vaccines of the invention in amounts effective to reduce amount or duration of bacterial shedding during infection. Animals that are suitable recipients of such vaccines include but are not limited to cattle, sheep, horses, pigs, poultry and other birds, cats, dogs, and humans. Methods of the invention utilize any of the vaccine compositions of the invention, and preferably, the vaccine comprises an effective amount of an attenuated, non-reverting mutant *Salmonella* bacterium in which one or more genes within the SPI2 region have been inactivated, either by deleting a portion of the gene(s), or, alternatively, by insertional mutation. In one aspect, methods utilize attenuated bacteria wherein an *ssa* gene is inactivated, and preferably the *ssa* gene is selected from the group consisting of *ssaT*, *ssaJ*, *ssaC*, and *ssaM*.

According to another aspect of the invention, the attenuated mutant *Salmonella* bacterium may further comprise a polynucleotide encoding a non-*Salmonella* polypeptide. Administration of the mutant bacteria or a vaccine



composition comprising the mutant bacteria thus provides a method of delivering an immunogenic polypeptide antigen to an animal.

Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently preferred embodiments thereof.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1, 2 and 3 show a schematic representation of the *S. typhimurium* nucleotide sequences flanking the internal deletions of the *ssaT*, *ssaJ* and *ssaC* genes, respectively, in the wild type and the mutant genome. The top sequence shows the nucleotides flanking the deletion points, while the lower sequence shows the DNA sequence present in the deleted locus. Small letters indicate nucleotides and capital letters specify amino acids.

Figure 4 shows the organization of SPI2 in *S. typhimurium*, which was assembled using sequences available on GenBank (Accession #'s AJ224892, Z95891, U51927, Y09357 and X99944). Predicted open reading frames are shown as open boxes with the orientation of the open reading frame indicated. The diagram is drawn to scale. Indicated at the bottom are the locations of *ssaC*, *ssaJ* and *ssaT* deletions.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides vaccines, or immunogenic compositions, comprising one or more species of attenuated mutant *Salmonella* bacteria in which one or more virulence genes, preferably genes within SPI2, have been deleted. An advantage of the vaccines of the present invention is that the live attenuated mutant bacteria can be administered as vaccines at reasonable doses, via a variety of different routes, and still induce protective immunity in the vaccinated animals. Another advantage is that mutant bacteria containing inactivations in two

different genes are non-reverting, or at least are much less likely to revert to a virulent state.

Risk of reversion can be assessed by passaging the bacteria multiple times (*e.g.*, 5 passages) and administering the resulting bacteria to animals. Non-reverting mutants will continue to be attenuated.

The examples herein demonstrate that inactivation of two or more genes within SPI2 does not further attenuate (or overattenuate) the bacteria compared to bacteria in which only a single gene has been inactivated. The examples also demonstrate that deletion of genes within SPI2 result in safe, efficacious vaccines as shown by observable reductions in adverse signs and symptoms associated with infection by wild type bacteria. The exemplary vaccines of the present invention have been shown to confer superior protective immunity compared to other vaccines containing live attenuated bacteria, *e.g.*, Salmo Shield®TD (Grand Laboratories, Inc.) and mutant *Salmonella* bacteria containing  $\Delta$ rfa K or  $\Delta$ cya  $\Delta$ crp mutations. The examples further demonstrate that a combination vaccine containing two or more strains of attenuated mutant *Salmonella* of different serotypes is efficacious and that inclusion of the different serotypes does not cause interference with the immune response.

When two or more genes within SPI2 are inactivated, the two genes may be from the same or different SPI2 “regions” (*e.g.*, the export machinery, effector proteins, and sensor/regulator regions). The genes may also be from the same or different functional groups as illustrated in Figure 4, which include the structural components, *ssa* (secretion system apparatus, *e.g.*, *ssaB*, *ssaC*, *ssaD*, *ssaE*, *ssaF*, *ssaG*, *ssaH*, *ssaI*, *ssaJ*, *ssaK*, *ssaL*, *ssaM*, *ssaN*, *ssaO*, *ssaP*, *ssaQ*, *ssaR*, *ssaS*, *ssaT*, and/or *ssaU*) secreted targets of the type III secretion system, *sse* (secretion system effector), the two-component sensor regulator system, *ssr* (secretion system regulator), and the chaperones for the secreted proteins, *ssc* (secretion system chaperone).

The nucleotide sequence of *ssaT* from *S. dublin* is set forth in SEQ ID NO: 1. The nucleotide sequence of *ssaT* from *S. typhimurium* is set forth in SEQ ID NO: 2. As used herein, “*ssaT*” includes SEQ ID NOS: 1, 2 and other *Salmonella*

species equivalents thereof, e.g., full length *Salmonella* nucleotide sequences that hybridize to the non coding complement of SEQ ID NO: 1 or 2 under stringent conditions (e.g., as described in Figure 4 of Shea et al., Proc. Nat'l. Acad. Sci. USA, 93:2593-2597 (1996), incorporated herein by reference), and full length *Salmonella* nucleotide sequences that have 90% sequence identity to SEQ ID NO: 1 or 2. *Salmonella* species equivalents can be easily identified by those of ordinary skill in the art and also include nucleotide sequences with, e.g. 90%, 95%, 98% and 99% identity to SEQ ID NO: 1 or 2.

The nucleotide sequence of *ssaJ* from *S. dublin* is set forth in SEQ ID NO: 3. The nucleotide sequence of *ssaJ* from *S. typhimurium* is set forth in NO: 4. As used herein, "*ssaJ*" includes SEQ ID NOS: 3, 4, and other *Salmonella* species equivalents thereof, e.g., full length *Salmonella* nucleotide sequences that hybridize to the non coding complement of SEQ ID NO: 3 or 4 under stringent conditions, and full length *Salmonella* nucleotide sequences that have 90% sequence identity to SEQ ID NO: 3 or 4. *Salmonella* species equivalents can be easily identified by those of ordinary skill in the art and also include nucleotide sequences with, e.g., 90%, 95%, 98% and 99% identity to SEQ ID NO: 3 or 4.

The nucleotide sequence of *ssaC* from *S. dublin* is set forth in SEQ ID NO: 5. The nucleotide sequence of *ssaC* from *S. typhimurium* is set forth in SEQ ID NO: 6. As used herein, "*ssaC*" includes SEQ ID NOS: 5, 6, and other *Salmonella* species equivalents thereof, e.g., full length *Salmonella* nucleotide sequences that hybridize to the non coding complement of SEQ ID NO: 5 or 6 under stringent conditions, and full length *Salmonella* nucleotide sequences that have 90% sequence identity to SEQ ID NO: 5 or 6. *Salmonella* species equivalents can be easily identified by those of ordinary skill in the art and also include nucleotide sequences with, e.g., 90%, 95%, 98% and 99% identity to SEQ ID NO: 5 or 6.

The nucleotide sequence of *ssaM* from *S. dublin* is set forth in SEQ ID NO: 30. The nucleotide sequence of *ssaM* from *S. typhimurium* is set forth in SEQ ID NO: 7. As used herein, "*ssaM*" includes SEQ ID NOS: 7, 30 and other *Salmonella* species equivalents thereof, e.g., full length *Salmonella* nucleotide sequences that

hybridize to the non coding complement of SEQ ID NO: 7 or 30 under stringent conditions, and full length *Salmonella* nucleotide sequences that have 90% sequence identity to SEQ ID NO: 7 or 30. *Salmonella* species equivalents can be easily identified by those of ordinary skill in the art and also include nucleotide sequences with, e.g., 90%, 95%, 98% and 99% identity to SEQ ID NO: 7 or 30.

The invention also contemplates that equivalent genes (e.g., greater than 80% homology) in other gram negative bacteria can be similarly inactivated to provide efficacious vaccines.

The nucleotide sequences of other genes in SPI2 can be assembled using sequences available on GenBank (e.g., Accession #'s AJ224892, Z95891, U51927, Y09357 and X99944) which cover SPI2.

As used herein, an "inactivated" gene means that the gene has been mutated by insertion, deletion or substitution of nucleotide sequence such that the mutation inhibits or abolishes expression and/or biological activity of the encoded gene product. The mutation may act through affecting transcription or translation of the gene or its mRNA, or the mutation may affect the polypeptide gene product itself in such a way as to render it inactive.

In preferred embodiments, inactivation is carried by deletion of a portion of the coding region of the gene, because a deletion mutation reduces the risk that the mutant will revert to a virulent state. For example, some, most (e.g., half or more) or virtually all of the coding region may be deleted (e.g., about 5% to about 100% of the gene, but preferably about 20% or more of the gene, and most preferably about 50% or more of the gene may be deleted). Alternatively, the mutation may be an insertion or deletion of even a single nucleotide that causes a frame shift in the open reading frame, which in turn may cause premature termination of the encoded polypeptide or expression of an completely inactive polypeptide. Mutations can also be generated through insertion of foreign gene sequences, e.g., the insertion of a gene encoding antibiotic resistance.

Deletion mutants can be constructed using any of a number of techniques well known and routinely practiced in the art. In one example, a strategy

using counterselectable markers can be employed which has commonly been utilized to delete genes in many bacteria. For a review, see, for example, Reyrat, *et al.*, *Infection and Immunity* 66:4011-4017 (1998), incorporated herein by reference. In this technique, a double selection strategy is often employed wherein a plasmid is constructed encoding both a selectable and counterselectable marker, with flanking DNA sequences derived from both sides of the desired deletion. The selectable marker is used to select for bacteria in which the plasmid has integrated into the genome in the appropriate location and manner. The counterselectable marker is used to select for the very small percentage of bacteria that have spontaneously eliminated the integrated plasmid. A fraction of these bacteria will then contain only the desired deletion with no other foreign DNA present. The key to the use of this technique is the availability of a suitable counterselectable marker.

In another technique, the *cre-lox* system is used for site specific recombination of DNA. The system consists of 34 base pair *lox* sequences that are recognized by the bacterial *cre* recombinase gene. If the *lox* sites are present in the DNA in an appropriate orientation, DNA flanked by the *lox* sites will be excised by the *cre* recombinase, resulting in the deletion of all sequences except for one remaining copy of the *lox* sequence. Using standard recombination techniques, it is possible to delete the targeted gene of interest in the *Salmonella* genome and to replace it with a selectable marker (*e.g.*, a gene coding for kanamycin resistance) that is flanked by the *lox* sites. Transient expression (by electroporation of a suicide plasmid containing the *cre* gene under control of a promoter that functions in *Salmonella* of the *cre* recombinase should result in efficient elimination of the *lox* flanked marker. This process would result in a mutant containing the desired deletion mutation and one copy of the *lox* sequences.

In another approach, it is possible to directly replace a desired deleted sequence in the *Salmonella* genome with a marker gene, such as green fluorescent protein (GFP),  $\beta$ -galactosidase, or luciferase. In this technique, DNA segments flanking a desired deletion are prepared by PCR and cloned into a suicide (non-replicating) vector for *Salmonella*. An expression cassette, containing a promoter

active in *Salmonella* and the appropriate marker gene, is cloned between the flanking sequences. The plasmid is introduced into wild-type *Salmonella*. Bacteria that incorporate and express the marker gene (probably at a very low frequency) are isolated and examined for the appropriate recombination event (*i.e.*, replacement of the wild type gene with the marker gene).

In order for a modified strain to be effective in a vaccine formulation, the attenuation must be significant enough to prevent the pathogen from evoking severe clinical symptoms, but also insignificant enough to allow limited replication and growth of the bacteria in the recipient. The recipient is a subject needing protection from a disease caused by a virulent form of *Salmonella* or other pathogenic microorganisms. The subject to be immunized may be a human or other mammal or animal, for example, farm animals including cows, sheep, pigs, horses, goats and poultry (*e.g.*, chickens, turkeys, ducks and geese) and companion animals such as dogs and cats; exotic and/or zoo animals. Immunization of both rodent and non-rodent animals is contemplated.

An "immunologically protective amount" of the attenuated mutant bacteria is an amount effective to induce an immunogenic response in the recipient that is adequate to prevent or ameliorate signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with wild type *Salmonella* bacteria. Either humoral immunity or cell-mediated immunity or both may be induced. The immunogenic response of an animal to a vaccine composition may be evaluated, *e.g.*, indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild type strain.

The protective immunity conferred by a vaccine can be evaluated by measuring, *e.g.*, reduction in clinical signs such as mortality, morbidity, temperature number and % of days of diarrhea, milk production or yield, average daily weight gain, physical condition and overall health and performance of the subject.

When a combination of two or more different serotypes of bacteria are administered, it is highly desirable that there be little or no interference among the

serotypes such that the host is not prevented from developing a protective immune response to one of the two or more serotypes administered. Interference can arise, e.g., if one strain predominates in the host to the point that it prevents or limits the host from developing a protective immune response to the other strain. Alternatively, one strain may directly inhibit the other strain.

In addition to immunizing the recipient, the vaccines of the invention may also promote growth of the recipient and/or boost the recipient's immunity and/or improve the recipient's overall health status. Components of the vaccines of the invention, or microbial products, may act as immunomodulators that may inhibit or enhance aspects of the immune system. For example, the vaccines of the invention may signal pathways that would recruit cytokines that would have an overall positive benefit to the host.

The vaccines of the present invention also provide veterinary and human community health benefit by reducing the shedding of virulent bacteria by infected animals. Either bacterial load being shed (the amount of bacteria, *e.g.*, CFU/ml feces) or the duration of shedding (*e.g.*, number of % of days shedding is observed) may be reduced, or both. Preferably, shedding load is reduced by about 10% or more compared to unvaccinated animals preferably by 20% or more, and/or shedding duration is reduced by at least 1 day, or more preferably 2 or 3 days, or by 10% or more or 20% or more.

While it is possible for an attenuated bacteria of the invention to be administered alone, one or more of such mutant bacteria are preferably administered in conjunction with suitable pharmaceutically acceptable excipient(s), diluent(s), adjuvant(s) or carrier(s). The carrier(s) must be "acceptable" in the sense of being compatible with the attenuated mutant bacteria of the invention and not deleterious to the subject to be immunized. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

Any adjuvant known in the art may be used in the vaccine composition, including oil-based adjuvants such as Freund's Complete Adjuvant and Freund's Incomplete Adjuvant, mycolate-based adjuvants (*e.g.*, trehalose dimycolate), bacterial

lipopolysaccharide (LPS), peptidoglycans (*i.e.*, mureins, mucopeptides, or glycoproteins such as N-Opaca, muramyl dipeptide [MDP], or MDP analogs), proteoglycans (*e.g.*, extracted from *Klebsiella pneumoniae*), streptococcal preparations (*e.g.*, OK432), Biostim™ (*e.g.*, 01K2), the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminum hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic® polyols, the Ribi adjuvant system (see, for example GB-A-2 189 141), or interleukins, particularly those that stimulate cell mediated immunity. An alternative adjuvant consisting of extracts of *Amycolata*, a bacterial genus in the order Actinomycetales, has been described in U.S. Patent No. 4,877,612. Additionally, proprietary adjuvant mixtures are commercially available. The adjuvant used will depend, in part, on the recipient organism. The amount of adjuvant to administer will depend on the type and size of animal. Optimal dosages may be readily determined by routine methods.

The vaccine compositions optionally may include vaccine-compatible pharmaceutically acceptable (*i.e.*, sterile and non-toxic) liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma.

The vaccine compositions can be packaged in forms convenient for delivery. The compositions can be enclosed within a capsule, caplet, sachet, cachet, gelatin, paper, or other container. These delivery forms are preferred when compatible with entry of the immunogenic composition into the recipient organism and, particularly, when the immunogenic composition is being delivered in unit dose form. The dosage units can be packaged, *e.g.*, in tablets, capsules, suppositories or cachets.

The vaccine compositions may be introduced into the subject to be immunized by any conventional method including, *e.g.*, by intravenous, intradermal,



intramuscular, intramammary, intraperitoneal, or subcutaneous injection; by oral, transdermal, sublingual, intranasal, anal, or vaginal, delivery. The treatment may consist of a single dose or a plurality of doses over a period of time.

Depending on the route of administration, suitable amounts of the  
 5 mutant bacteria to be administered include  $\sim 10^9$  bacteria or less, provided that an adequate immunogenic response is induced by the vaccinee. Doses of  $\sim 10^{10}$  or less or  $\sim 10^{11}$  or less may be required to achieve the desired response. Doses significantly higher than  $\sim 10^{11}$  may not be commercially desirable.

Another aspect of the invention involves the construction of attenuated  
 10 mutant bacteria that additionally comprise a polynucleotide sequence encoding a heterologous polypeptide. For example, for *Salmonella*, a “heterologous” polypeptide would be a non-*Salmonella* polypeptide not normally expressed by *Salmonella* bacteria. Such attenuated mutant bacteria can be used in methods for delivering the heterologous polypeptide or DNA. For example, *Salmonella* could be engineered to  
 15 lyse upon entry into the cytoplasm of a eukaryotic host cell without causing significant damage, thereby becoming a vector for the introduction of plasmid DNA into the cell. Suitable heterologous polypeptides include immunogenic antigens from other infectious agents (including gram-negative bacteria, gram-positive bacteria and viruses) that induce a protective immune response in the recipients, and expression of  
 20 the polypeptide antigen by the mutant bacteria in the vaccine causes the recipient to be immunized against the antigen. Other heterologous polypeptides that can be introduced using the mutant *Salmonella* include immunomodulatory molecules e.g., cytokines or “performance” proteins such as growth hormone, GRH, and GDF-8.

Example 1 addresses the construction of attenuated mutant *Salmonella*  
 25 bacteria containing defined single or double deletions in the *ssaT*, *ssaJ*, *ssaC*, *rfaK* and *glnA* genes; and construction of mutant *Salmonella* containing inactivating insertions. Example 2 addresses the results of safety and efficacy testing of animals inoculated with these attenuated mutant *Salmonella* bacteria.

### Example 1

#### Construction of Salmonella Mutants Containing Single and Double Deletions of Selected Genes: *ssaT*, *ssaJ*, *ssaC*, *rfaK* and *glnA*

##### A. Construction of pCVD442::Δgene plasmids.

For each of the *S. typhimurium* and *S. dublin* *ssaT*, *ssaJ*, *ssaC*, *rfaK* and *glnA* genes, positive selection suicide vectors based on the plasmid pCVD442 [Donnenberg and Kaper, Infect Immun 59:4310-17 (1991)] were constructed that contained a portion of the 5' and 3' chromosomal regions flanking each gene but with substantial internal deletions (typically >95%) within the gene itself. Gene splicing by overlap extension ("gene SOEing" [Horton et al., Biotechniques 8:528-535 (1990)]) was used to generate DNA fragments which were complementary to the gene to be deleted, but which lacked the majority of the internal nucleotide sequence. The plasmids containing these internally deleted genes were designated pCVD442::Δ*ssaT*, pCVD442::Δ*ssaJ*, pCVD442::Δ*ssaC*, pCVD442::Δ*rfaK*, and pCVD442::Δ*glnA*, respectively. These vectors were then used to generate *S. typhimurium* and *S. dublin* deletion mutants by allelic exchange. Plasmids containing the *S. dublin* deleted genes was used to produce the deletions in *S. dublin*, and plasmids containing the *S. typhimurium* sequences was used to produce the deletions in *S. typhimurium* (see Example 1B below).

In brief, two sets of PCR primers were designed to synthesize approximately 600 bp fragments that are complementary to the DNA flanking the 5' and 3' sides of the desired gene. Primers A and D (Table 1) contain chromosomal sequence upstream and downstream, respectively, of the desired gene and each also contains the nucleotide sequence for a desired restriction endonuclease site. Primer B spans the upstream junction between the sequences immediately flanking the 5' side of the gene and the gene itself and includes some a portion of the 5' end of the gene (in some cases, only the stop codon). Similarly, primer C spans the downstream junction between the sequences immediately flanking the 3' side of the gene and the gene itself, and includes a portion of the 3' end of the gene (in some cases, only the start codon).

PCR reactions with *S. typhimurium* or *S. dublin* genomic DNA and either primers A and B or primers C and D were performed, yielding PCR products (designated fragments AB and CD, respectively) of approximately 600 bp with sequences corresponding to the upstream or downstream flanking regions of the desired gene, respectively. Each AB or CD fragment also contained the desired restriction site (*Xba*I for *ssaT*, *ssaJ*, *ssaC*, and *glnA* and *Sal*I for *rfaK*). A second PCR reaction using fragments AB and CD with primers A and D was then performed, yielding a PCR product designated fragment AD. Fragment AD is complementary to the nucleotide sequence surrounding the targeted gene, but contains essentially a complete deletion of the targeted sequences (> 95% deletion) for *ssaS*, *ssaT*, *rfaK*, and *glnA*, and a deletion of the C-terminal half (~50% deletion) for *ssaJ* (see Figs. 1-3). The resulting PCR product for each of the *S. dublin* or *S. typhimurium* *ΔssaT*, *ΔssaJ*, *ΔssaC*, *ΔrfaK*, and *ΔglnA* genes was then cloned through various vectors and host strains and finally inserted into the multiple cloning site of vector pCVD442 in host strain SM10λ*pir*.

Figures 1, 2 and 3 show the nucleotide sequences flanking the junctions of the *ssaT*, *ssaJ* and *ssaC* genes in the wild type and the mutant genome. These deletions were engineered to remove as much of the open reading frame as feasible. In the case of the *ssaC* (1052 bp) and *ssaT* (779 bp) genes the entire open reading frame of each was deleted, leaving only the start and stop codons (6 bp) after the deletion was generated. Only the C-terminal half of the *ssaJ* gene could be removed when the deletion was created. This reduced the size of the gene from 749 bp to 324 bp.

Figure 4 shows the genetic arrangement of the SPI2 locus in *S. typhimurium*. This is a compilation of the SPI2 region of *S. typhimurium* assembled using sequences available on GenBank (Accession #'s AJ224892, Z95891, U51927, Y09357 and X99944). Also shown are the positions of the deletions constructed in the *ssaC*, *ssaJ* and *ssaT* genes.

Table 1. Primers used in construction of modified *S. typhimurium* and *dublin* genes\*.

Gene	Primer Number (Letter)	Sequence ID NOs:	Primer Sequence	Restriction Site
<i>ssaT</i>	642 (A)	10	5'-GCCAATCTAGAAATTATTTTCGGAAATTTGATAAA-3'	<i>XbaI</i>
	643 (B)	11	5'-AGGCTGTTCTGTTTCTCGCTCACATTCAACCATGCTCTCCAATTCGTA-3'	----
	644 (C)	12	5'-TACGAAATTGGAGAGCATGTTGAATGTGAGCGAGAAAAACAGAACAGCCT-3'	----
	645 (D)	13	5'-GCCAATCTAGATCTTTTCTAATCTTATAATTTG-3'	<i>XbaI</i>
<i>ssaJ</i>	638 (A)	14	5'-GCCAATCTAGACTGCAGAACCGAGCCAGGAGCAA-3	<i>XbaI</i>
	639 (B)	15	5'-CACCTCGGATCAGGTCGGTCTATAAAAAATTAACTTCTGCTGTT-3'	----
	640 (C)	16	5'-AACAGCAGAAAGATTAAATTTTATGAGCCGACCTGATCCCGAGGTG-3'	---
	646 (D)	17	5'-GCCAATCTAGAGAAGATAATCTCGGTAAGAGAAAGT-3'	<i>XbaI</i>
<i>ssaC</i>	634 (A)	18	5'-GCCAATCTAGATTCAAATGTAAGTTTATGTCAAT-3'	<i>XbaI</i>
	635 (B)	19	5'-TTTATCCAGCACAGCCTGGATATTACATTTTATACCCACCCGGAATAAAAG-3'	----
	647 (C)	20	5'-TTATTCGGTGGGTATAAAATGTAATATCCAGGCTGTGCTG-3'	----
	637 (D)	21	5'-GCCAATCTAGATTCCCGGCATCAACAAAATAAACT-3'	<i>XbaI</i>
<i>rfaK</i>	882 (A)	22	5'-GCCAAGTCGACATAGTAGGTGTTCTGTGGGCAATA-3'	<i>SalI</i>
	883 (B)	23	5'-TTCTGGATTATAGCTATTATGATTGTTTGATAAGTGATTGATCTCTGA-3'	----

Gene	Primer Number (Letter)	Sequence ID NOs:	Primer Sequence	Restriction Site
	884 (C)	24	5'-TCAGGACTCAATCACTTATCAAAACAATCATAATAGCTATAATCCAGAA-3'	----
	885 (D)	25	5'-GCCAAGTCGACGTGTACGAACAGGCTTCAGTGGAT-3'	<i>SalI</i>
<i>gln4</i>	886 (A)	26	5'-GCCAATCTAGATCAGGCATTAGAAATAGCGCGTTA-3'	<i>XbaI</i>
	887 (B)	27	5'-ATTTTAAATATACGATTAAACGCTCAAAACATTTCGCTTCTCAAAGA-3'	----
	888 (C)	28	5'-TCTTTGAAAGAGGCCAAAATGTTGAGCGTTTAATCGTATATTAAAAAT-3'	----
	889 (D)	29	5'-GCCAATCTAGATGCTCTGACTCAGACGCGCTGG-3'	<i>XbaI</i>

\*PCR primers used in generating the left (5') and right (3') flanking regions of the *ssaT*, *ssaJ*, *ssaC*, *rfaK* and *gln4* genes. Primers A/B and C/D are the 5' and 3' primer sets, respectively, for that gene. Primers A and D are the primers that are the furthest upstream and downstream from that gene and were designed to incorporate the restriction sites indicated into the PCR product.

The *S. dublin* and *S. typhimurium* genes are similar enough that the same primers could be used for both serotypes.

### B. Construction of Deletion Mutants of *S. typhimurium* and *S. dublin*

The pCVD442:: $\Delta$ gene plasmids constructed in Example 1A above were used to produce deletion mutants by homologous recombination with appropriate *Salmonella* strain i.e., a plasmid containing the *S. dublin* deleted gene was used to produce the deletion in *S. dublin*, and a plasmid containing the *S. typhimurium* sequences was used to produce the deletion in *S. typhimurium*. The plasmid pCVD442 is a positive selection suicide vector. It contains the origin of replication for R6K plasmids (*ori*), the mobilization gene for RP4 plasmids (*mob*), the gene for ampicillin resistance (*bla*), the *sacB* gene from *B. subtilis*, which encodes the gene for levan sucrase and a multiple cloning site.

The plasmid pCVD442 can be maintained extrachromosomally only in bacterial strains producing the  $\pi$  protein, the *pir* gene product (e.g. *E. coli* SM10 $\lambda$ *pir* or DH5 $\alpha$  $\lambda$ *pir*). Introduction of a pCVD442 based vector into a nonpermissive host strain (*S. typhimurium* or *S. dublin*), by conjugation and selection on Ap (ampicillin) and Nal (nalidixic acid) containing medium, allows the isolation of Ap<sup>R</sup> merodiploid isolates in which the plasmid has integrated into the genome of the target strain by homologous recombination with the wild type gene.

In brief, *E. coli* strain SM10 $\lambda$ *pir* (*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu km*)[(Donnenberg and Kaper, Infect Immun 59:4310-17 (1991)] carrying the pCVD442 plasmids with the *S. typhimurium* or *S. dublin*  $\Delta$ *ssaT*,  $\Delta$ *ssaJ*,  $\Delta$ *ssaC*,  $\Delta$ *rfaK* or  $\Delta$ *glnA* genes (designated SM10 $\lambda$ *pir*/pCVD442:: $\Delta$ gene) were mated with NaI<sup>R</sup> *S. typhimurium* MK315N or *S. dublin* B94-058N, and recombinants were selected on Ap and Nal. Both MK315N and B94-058N are spontaneous NaI<sup>R</sup> strains prepared by plating the respective parent strains on LB agar containing 50 $\mu$ g/ml NaI (clinical isolates from a bovine and a human subject, respectively). The Ap<sup>R</sup> NaI<sup>R</sup> recombinants recovered must have the plasmid integrated into the chromosome because the plasmid cannot be maintained extrachromosomally. This results in the

formation of a merodiploid strain that contains the pCVD442:: $\Delta$ gene plasmid integrated into that gene locus on the chromosome.

The Ap<sup>R</sup> Nal<sup>R</sup> *S. typhimurium* MK315N::pCVD442:: $\Delta$ gene and *S. dublin* B94-058N::pCVD442:: $\Delta$ gene recombinants were then grown under  
 5 non-selective conditions followed by growth on LA (- sucrose) and TYES (+ sucrose) agar. In the absence of selection pressure a spontaneous recombination event can occur in which the pCVD442 plasmid and either the wild-type gene or the deleted gene are excised from the chromosome. Cells retaining the pCVD442 plasmid were  
 10 counterselected on TYES agar by the toxic products produced from the breakdown of sucrose by levan sucrose, encoded by the *sacB* gene. Consequently, the number of colonies on TYES agar is significantly reduced relative to the number on LA. After confirming the Ap<sup>S</sup> phenotype of the isolated colonies on the TYES agar, the recombinants were analyzed by PCR to determine whether the wild-type gene or the  
 15 deleted gene had been retained.

In initial experiments, the donor and recipient were mated for 5 hrs. on  
 LB agar and then selected on LB agar containing Nal (20 or 100  $\mu$ g/ml) and Ap (20  
 or 100  $\mu$ g/ml). While heavy growth appeared on the initial selection plate few, if any,  
 of the isolated colonies could be confirmed as Ap<sup>R</sup> Nal<sup>R</sup>. The inability to isolate  
 recombinant growth was likely due to the growth of the recipient as a result of the  
 20 degradation of ampicillin by the release of  $\beta$ -lactamase from the donor cells. To  
 overcome this problem, mating and selection conditions were designed that favored  
 the recombinants and selected against the donor and recipient strains. Specifically,  
 recipient and donor strains were mated overnight on LB agar or modified M9 agar  
 (Difco Laboratory, Detroit, MI), followed by enrichment of recombinants by growth  
 25 in selective (Nal and Ap (75 $\mu$ g/ml)) LB broth (Difco Laboratory, Detroit, MI), and  
 isolation on selective (Nal and Ap (75 $\mu$ g/ml)) agar medium. Mating on modified M9  
 agar allowed conjugation to occur, but limited replication, which reduced the number  
 of donor and recipient cells introduced to the selection broth. Growth to early  
 logarithmic phase in selection broth favored the replication of the recombinants but  
 30 not the donor and recipient strains. Subsequent selection on LB agar Nal Ap (75

µg/ml each) further favored the recombinants over the donor and recipient, which was confirmed when almost all isolated colonies were Ap<sup>R</sup> Nal<sup>R</sup>. This procedure yielded merodiploid *S. typhimurium* or *S. dublin* recombinants carrying the appropriate plasmid pCVD442::Δgene inserted into the genome.

5 Meridiploid isolates were then grown under non-selective conditions to late logarithmic phase and inoculated to LB agar and TYES agar. During non-selective growth a spontaneous recombination event can occur between the duplicated sequences in the merodiploid state, leaving a copy of either the wild type or duplicated gene in the chromosome. Growth on sucrose (TYES) selects against those  
10 cells which have not undergone the second recombination event because the products of levan sucrase, encoded by the *sacB* gene on the pCVD442 plasmid, are toxic to gram-negative cells. Consequently, the number of colonies on TYES agar is much lower than on LA. In our hands, it was critical to incubate the TYES plates at room temperature for the selection to be successful. Incubation at higher temperatures (30°  
15 or 37°C) did not reduce the number of colonies on TYES relative to LA indicating that selection for pCVD442-negative cells did not occur.

TYES-grown colonies were streaked for single colony and the Nal<sup>R</sup> Ap<sup>S</sup> phenotype confirmed. PCR analysis of the genomic DNA of the colonies using the appropriate Primers A and D described above for each gene was then performed to  
20 determine whether the deleted or wild type gene had been retained in the chromosome. For *ssaT*, a PCR product of 1206 bp (vs. 1980 bp for wild type gene) indicated that the gene had been deleted. For *ssaJ*, a PCR product of 1074 bp (vs. 1500 bp for wild type gene) indicated that the gene had been deleted. For *ssaC*, a PCR product of 1229 bp (vs. 2275 bp for wild type gene) indicated that the gene had  
25 been deleted. For *rfaK*, a PCR product of 1300 bp (vs. 2400 bp for wild type gene) indicated that the gene had been deleted. For *glnA*, a PCR product of 1161 bp (vs. 2411 for wild type gene) indicated that the gene had been deleted.

Although theoretically 50% of these isolates should carry the deleted gene, the actual percentage of isolates carrying the deleted gene was quite variable,  
30 ranging from 1.4% (1 out of 73 isolates) for the *S. dublin glnA* gene to 80% (4 out of



5) for the *S. dublin ssaJ* gene, suggesting that the excision of the gene was not random. Deletion mutants were confirmed to be *S. typhimurium* or *S. dublin* by serological tests.

### C. Construction of Double Deletion Mutants of *S. typhimurium* and *S. dublin*

5 *Salmonella* double deletion mutants were constructed in the same fashion as the *Salmonella* single deletion mutants using *E. coli* and *Salmonella* strains described above. In brief, *E. coli* SM10 $\lambda$ pir donor strains carrying the pCVD442 plasmid with the deleted *ssaT* gene ( $\Delta$ *ssaT*) from either *S. typhimurium* or *S. dublin* were mated with *S. dublin* B94-058N or *S. typhimurium* MK315N mutants with either  
10 the deleted *ssaC* or *ssaJ* gene ( $\Delta$ *ssaC* and  $\Delta$ *ssaJ*), and Ap<sup>R</sup> exconjugants were selected. Introduction of the plasmid into the nonpermissive *Salmonella* host and selection of Ap<sup>R</sup> colonies allowed the isolation of merodiploid recombinants in which the pCVD442 plasmid was integrated into the genome by homologous recombination with the wild type gene. Subsequent growth of the merodiploid recombinants to late  
15 logarithmic phase under non-selective conditions allowed a spontaneous recombination event to occur between the duplicated sequences in the merodiploid genome, leaving a copy of either the wild type or deleted gene in the chromosome. Colonies grown under these non-selective growth conditions were then inoculated into sucrose containing medium, which selected against those cells which have not  
20 undergone the second recombination event. Isolated sucrose resistant colonies of *S. typhimurium* were analyzed by PCR to identify the desired *ssaT* deletion mutant.

Despite assaying numerous sucrose resistant colonies by PCR, an *S. dublin ssaT* deletion mutant could not be identified. The putative B94-058N::*AssaCssaT* and B94-058N::*AssaJAssaT* recombinants were identified by  
25 colony hybridization. The *E. coli* SM10 $\lambda$ pir donor strains carrying the pCVD442 plasmid with the deleted *ssaT* gene ( $\Delta$ *ssaT*) from *S. dublin* were mated with *S. dublin* B94-058N mutants with either the deleted *ssaC* or *ssaJ* gene ( $\Delta$ *ssaC* and  $\Delta$ *ssaJ*) and selected for sucrose resistant colonies as described above. These colonies were then grown on selective (Ap and NaI) media and then lifted onto nitrocellulose (Schleicher

& Schuell nitrocellulose BA85; Schleicher and Schuell, Keene, NH). The nitrocellulose blot was processed as described in Davis et al., Basic methods in molecular biology, Elsevier, New York (1986) except that the second and third buffers used were 1 M Tris, pH 7.6, and 1.5 M NaCl-0.25 M Tris, pH 7.6, respectively. The bacterial DNA was crosslinked to the nitrocellulose using a Stratagene UV Stratalinker 1800 (Stratagene, La Jolla, CA) using the auto crosslink setting (1200  $\mu$ J X 100). The blot was prehybridized, hybridized, washed and developed according to the Genius system instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN), using the manufacturer's instructions. Internal sequences of the *ssaT* gene was used as the probe during the hybridization step. The probe was generated by PCR using DEL-1680 (5'-TGGCTTTTATTCGACCATTGAGCCTTTC-3' (SEQ ID NO: 8)) and DEL-1681 (5'-TTTATCGCTTTCACCAAATAGTGATG-3' (SEQ ID NO: 9)) as the primers and suspended B94-058N colonial growth as the DNA template. The DNA probe was labeled by incorporating digoxigenin into the abbreviated *ssaT* gene using the DIG-High Primer kit (Boehringer Mannheim Biochemicals). The colonies which did not yield a positive signal, putative *ssaT* deletion mutants, were analyzed for the presence of the wild type or deleted *ssaT* gene by PCR using suspended colonial growth and primers A and D (see Example 1A) for the *ssaT* gene as described above.

To isolate the desired double deletion mutants it was necessary to analyze recombinants after non-selective growth using either PCR (*S. typhimurium* recombinants) or colony hybridization followed by PCR (*S. dublin* recombinants). In theory the excision of the wild type and deleted gene should occur randomly and at an equal frequency resulting in 50% of the recombinants carrying the deleted gene. As shown in Example 1B, however, the percentage of isolates with the deleted gene was quite variable. The frequency in which the deleted  $\Delta$ *ssaT* gene was recovered in the putative *S. typhimurium* MK315N::*ΔssaJΔssaT*, *S. typhimurium* MK315N::*ΔssaCΔssaT*, *S. dublin* B94-058N::*ΔssaCΔssaT* and *S. dublin* B94-058N::*ΔssaJΔssaT* recombinants was 50% (4 out of 8), 12.5% (1 out of 8), 3.8% (8 out of 209) and 8.9% (14 out of 158), respectively.

PCR analysis of the genomic DNA of putative *S. typhimurium* and *S. dublin* double deletion mutants (using the appropriate Primers A and D described above) confirmed the presence of both the *ssaC* and *ssaT* or *ssaJ* and *ssaT* deleted genes. Deletion mutants were confirmed to be *S. typhimurium* or *S. dublin* by serological tests. *S. typhimurium* and *S. dublin* mutants were shown to agglutinate in *Salmonella* O Group B and *Salmonella* O Group D<sub>1</sub> antisera (Difco Laboratory, Detroit, MI), respectively, as expected.

#### D. Construction of *S. choleraesuis* mutants

*S. choleraesuis* mutants were constructed using the STM process generally described in U.S. Patent No. 5,876,931, incorporated herein by reference. Briefly, each insertional mutation produced carries a different DNA signature tag, which allows mutants to be differentiated from each other. The tags comprise 40-bp variable central regions flanked by invariant "arms" of 20-bp which allow the central portions to be co-amplified by PCR. Tagged mutant strains are assembled in microtiter dishes, then combined to form the "inoculum pool" for infection studies. At an appropriate time after inoculation, bacteria are isolated from the animal and pooled to form the "recovered pool." The tags in the recovered pool and the tags in the inoculum pool are separately amplified, labeled, and then used to probe filters arrayed with the different tags representing the mutants in the inoculum. Mutants with attenuated virulence are those with tags that give hybridization signals when probed with tags from the inoculum pool but not when probed with tags from the recovered pool. STM allows a large number of insertional mutant strains to be screened simultaneously in a single animal for loss of virulence. Using this method, insertional mutants of *S. choleraesuis* containing a mini-tn5 transposon interrupting the particular gene were generated. Portions of the gene surrounding each transposon were sequenced to identify the insertion site by alignment of the sequence with the corresponding sequence of known *S. typhimurium* genes. The mini-tn5 transposon creating the  $\Delta$ *ssaM* mutation was located between nucleotide positions 126 and 127

in SEQ ID NO: 7, and the mini-tn5 transposon producing the *AssaJ* mutation was located between nucleotide positions 209 and 210 in SEQ ID NO: 4.

### E. Characterization of Single and Double SPI2 Mutants

Data showed that certain of the single and double deletion mutants, specifically *AssaC* and *AssaCAssaT*, are less invasive than the wild-type and other mutants. This may contribute to slightly better efficacy. It may be that mutants, e.g., *AssaC* mutants, that remain associated with intestinal mucosa for longer periods of time yet have a reduced ability to invade host epithelial cells, are better able to elicit strong mucosal immunity.

## EXAMPLE 2

### Safety and Efficacy of Single and Double Deletion SPI2 Mutants

#### A. Efficacy of a *S. choleraesuis AssaC* mutant as a vaccine in swine

(Trial No. 704-7923-I-MJK-96-008)

The safety and efficacy of a live attenuated *S. choleraesuis AssaC* mutant as a vaccine was determined in swine (3-4 week old pigs). The pigs (8 pigs per group) were vaccinated either orally via the drinking water or intramuscularly (IM), at a dose of about  $\sim 1 \times 10^9$  CFUs/pig. For oral vaccination, 10 ml of the lab grown culture of the vaccine (grown generally as described in Example 2.D. below) was diluted 1:4 in ddH<sub>2</sub>O. 40 ml of this mixture was further diluted by adding 960 ml of sterile ddH<sub>2</sub>O; 100 ml of this final mixture was given to pigs orally via a waterer (pigs drank approximately 50 ml of water, giving a final vaccine dose of  $\sim 2.6 \times 10^8$  per pig). For the IM vaccination, 27 ml of the 1:4 diluted vaccine culture was added to 3 ml of sterile WFI; 2.5 ml of this mixture was administered intramuscularly to each animal giving a final dose of  $\sim 3.0 \times 10^8$  per pig. The pigs were monitored daily for temperature, body weight, fecal consistency scores, physical condition, and mortality. Animals were also monitored for shedding of the vaccine and challenge

organisms. All animals were necropsied at termination of the trial and tissues were cultured for the challenge organism.

Following vaccination, no adverse clinical signs of disease were observed in animals vaccinated orally or IM with the *ΔssaC* mutant except for a short-term elevation in temperature. The pigs were then rechallenged with the highly virulent wild type *S. choleraesuis*, which was a field isolate obtained from a case of salmonellosis, at 28 days post-vaccination. Results showed that administration of the *ΔssaC* mutant by either oral or intramuscular routes was safe and efficacious against experimentally induced salmonellosis. Non-vaccinates exhibited severe pyrexia, which was accompanied by watery diarrhea, anorexia, dehydration, and death. In contrast, animals vaccinated with the *ΔssaC* vaccine constructs were resistant to infection. In these animals, there was a statistically significant reduction in both the severity and duration of morbidity, mortality, days of inactivity, and shedding of the challenge organism.

#### **B. Efficacy of a *S. choleraesuis* *ΔssaM* or *S. choleraesuis* *ΔssaC* mutants as vaccines in swine** (704-7923-I-MJK-96-012)

The safety and efficacy of a live attenuated *S. choleraesuis* *ΔssaM* or a *S. choleraesuis* *ΔssaC* mutant as a vaccine was determined in swine (3-4 week old pigs). Eight pigs were vaccinated orally via the drinking water at a dose of about  $\sim 1 \times 10^9$  CFUs/pig. For oral vaccination, a lab grown vaccine culture (grown generally as described in Example 2.D. below) was diluted 1:4 in ddH<sub>2</sub>O; 40 ml of this mixture was added to 960 ml of sterile ddH<sub>2</sub>O; and 100 ml of this final mixture was given to pigs via a waterer. The number of CFUs per ml was determined by performing serial 10-fold dilutions of the final formulation, and plating on agar. The dose per pig was then determined by multiplying the number of CFUs/ml by the number of mls of water consumed by the animal (each pig drank approximately 50 ml of water), giving a final vaccine dose of  $\sim 1 \times 10^9$  CFUs/pig. Baseline values for body temperatures, fecal consistency, and physical condition for each animal were collected during the

four days immediately prior to vaccination, and were compared to post-vaccination values to assess the safety of each vaccine. The pigs were monitored daily for temperature, body weight, fecal consistency scores, physical condition, and mortality. Animals were also monitored for shedding of the vaccine and challenge organisms.

5 All animals were necropsied at termination of the trial and tissues were cultured for the challenge organism.

Following vaccination, no adverse clinical signs of disease were observed in animals vaccinated orally with either SPI2 mutant except for a short-term elevation in temperature. The pigs were then rechallenged with the highly virulent

10 wild type *S. choleraesuis*, which was a field isolate obtained from a case of salmonellosis, at 28 days post-vaccination. Results showed that administration of either SPI2 mutant by oral routes was safe and efficacious against experimentally induced salmonellosis. Non-vaccinates exhibited severe pyrexia, which was accompanied by watery diarrhea, anorexia, dehydration, and death. In contrast,

15 animals vaccinated with the *AssaC* or *AssaM* vaccine constructs were resistant to infection. In these animals, there was a significant reduction in both the severity and duration of morbidity, mortality, days of inactivity, and shedding of the challenge organism.

### **C. Efficacy of a *S. choleraesuis* *AssaJ* or a *S. choleraesuis* *AssaC* mutant as vaccines in swine**

704-7923-I-MJK-97-004

The safety and efficacy of a live attenuated *S. choleraesuis* *AssaJ* or a *S. choleraesuis* *AssaC* mutant as a vaccine was determined in swine (56 male & female crossbred pigs, 18-24 days of age at vaccination). Baseline temperatures and clinical scores (mortality, morbidity, diarrhea, shedding of bacteria and average daily

25 weight gain) were recorded on Days 1-4. The pigs were vaccinated orally via the drinking water on Day 4 at a dose of about  $\sim 1 \times 10^9$  CFUs/pig as described above in Example 2B. The pigs were monitored daily for clinical symptoms (% mortality, % morbidity, % diarrhea days, % shedding days, and average daily gain, determined as

described in Example 2.D. below) for 21 days post-vaccination (Days 5-25), of which Days 22-25 were considered a baseline before challenge with wild type bacteria. The pigs were then challenged with a highly virulent wild type *S. choleraesuis*, which was a field isolate obtained from a case of salmonellosis, orally via feed (following a 24 hour fast) at 21 days post-vaccination (Day 25). The pigs continued to be monitored for clinical symptoms for a further 21 days post-challenge (Days 26-46). Results post-vaccination (and pre-challenge) are displayed in Table 2 below. Results post-challenge are displayed in Table 3 below. The results showed that oral administration of each of these two mutants as a vaccine was safe and efficacious against experimentally induced salmonellosis.

**Table 2.** Lack of clinical signs in vaccinates showing the safety of *S. choleraesuis* vaccine post-vaccination.

Vaccine	Time	N =	% Mortality	% Morbidity	% Diarrhea Days	% Shedding Days	Ave. Daily Gain
None	pre	8	0	0	0	0	0.48
	post		0	0	3.3	0	
$\Delta$ ssaC	pre	8	0	0	3.1	0.0	0.39
	post		0	0	4.0	9.7	
$\Delta$ ssaJ	pre	8	0	0	2.1	0.0	0.38
	post		0	1.6	1.9	13.5	

**Table 3.** Reduction in clinical signs in vaccinates post-challenge showing the efficacy of *S. choleraesuis* vaccine.

Vaccine	N =	% Mortality	% Morbidit y	% Diarrhea Days	% Shedding Days	Ave. Daily Gain
None	8	0	13.9	34.5	54.8	0.44
<i>ΔssaC</i>	8	0	5.2	11.1	14.4	0.69
<i>ΔssaJ</i>	8	0	10.7	10.7	18.3	0.61

5 **D. Efficacy of a *S. dublin* *ΔssaC* or a *S. dublin* *ΔssaJ* or a *S. dublin* *ΔssaT* mutant as vaccines in cattle**

(2051-7923-I-MJK-98-004)

The safety and efficacy of a live-attenuated *S. dublin* *ΔssaC* or *S. dublin* *ΔssaJ* or *S. dublin* *ΔssaT* mutant as vaccines was determined in cattle (36 male and female calves, 10-14 days of age at vaccination). Live-attenuated *S. dublin* strains were revived from stock cultures by streaking onto blood agar. After incubation for 18-24 hr at 37°C, colonies from a heavy growth area were swept with a sterile loop and inoculated into LB broth. After 14 hrs of static incubation at 37°C, 1.0 ml of this culture was used to inoculate 22.5 ml of fresh LB broth in 250 ml sterile polycarbonate Erlenmeyer flasks. After 6 hrs of static incubation at 37°C, 2.5 ml of the resulting undiluted broth culture was added to 3.0 liters of milk replacer for administration to each calf. Dilution and viable plate count on blood agar determined "Numbers of Viable Bacteria" for each strain at the time of preparation. Each vaccine was maintained at room temperature and delivered to animals as soon as possible after preparation (within ~30 minutes). Baseline temperatures and clinical scores (mortality, physical condition, inactivity, diarrhea (fecal score), and shedding of bacteria) were recorded on Days 1-4. The calves were vaccinated orally via the milk replacer on Day 4 with either wild type or a mutant bacteria at a dose of about ~1x10<sup>9</sup> CFUs/calf. For oral vaccination, 1 ml of the lab grown vaccine culture was inoculated in the calf's milk replacer. The number of CFUs/ml was determined as described above in Example 2B. Because each calf consumed its entire amount of



milk replacer on the day of vaccination, the number of CFUs per animal was the same as the number of CFUs/ml of culture.

The calves were monitored daily for clinical symptoms (% mortality, physical condition, % inactive days, fecal score, and 5 shedding days) for 28 days post-vaccination (Days 5-32), of which Days 29-32 were considered a baseline before challenge with wild type bacteria. If a calf died during the period of interest, it was assigned a score of “1” for the mortality variable, otherwise, the mortality variable assigned was “0”. The physical condition was scored on a scale of 1 to 5, where “1” was a healthy, active animal with normal hair-coat; “2” was a mildly depressed animal that was intermediate in activity and had a rough hair-coat; “3” was a moderately to severely depressed animal that was inactive/lethargic and/or gaunt irrespective of hair-coat; “4” was a moribund animal; and “5” was a dead animal. If a calf died, the physical condition was assigned a “5” for the day of death (or the following day depending on the time of death), and missing values thereafter. The average physical condition was taken as the average of the daily scores within the period of interest for each calf. The average physical scores were then used to calculate the rescaled score in the following way: the rescaling score =  $100 \times (\text{average physical condition score} - 1)/4$ . This converts the 1-5 scale into a 0-100 scale. The % inactive days score was determined by calculating the percent of days during the period of interest that a calf had a score of greater than 2 on the physical condition score. The fecal score was scored on a scale of 1-4 where “1” is normal, solid formed or soft with form; “2” is soft unformed; “3” is watery with solid material; and “4” is profuse watery/projectile with little or no solid material. The % shedding days was calculated as the percent of days during the period of interest that a calf had a rectal swab positive for *Salmonella*.

The calves were then challenged with a highly virulent, heterologous wild type *S. dublin*, which was a field isolate obtained from a case of bovine salmonellosis, at 28 days post-vaccination (Day 32). The calves continued to be monitored for clinical symptoms for a further 14 days post-challenge (Days 33-46). Results post-vaccination (and pre-challenge) are displayed in Table 4 below. Results post-challenge are displayed in Table 5 below. Necropsy was performed on Day 46 or

at death, and tissue and fecal samples were obtained for culture of the challenge organism. The data from culturing of tissue (>2g) or fecal (>2g) samples showed that there was a reduction of the challenge strain in the tissues from animals vaccinated with the SPI2 mutants compared to the naive controls, and that oral administration of each of these three mutants as a vaccine was safe and efficacious against experimentally induced salmonellosis. Protective effects seen with these SPI2 mutants were better than those observed with  $\Delta$ cya  $\Delta$ crp mutants.

**Table 4.** Lack of clinical signs in vaccinates showing safety of *S. dublin* vaccines post-vaccination.

Vaccine /Strain	Time	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
None	pre	6	0	0.0	0	1.2	0.0
	post (1-28)		0	0.0	0	1.1	0.0
wild-type	pre	6	0	0.0	0	1.3	0.0
	post (1-28)		16.7	5.4	5.6	1.4	64.1
$\Delta$ ssaC	pre	6	0	0.0	0	1.0	0.0
	post (1-28)		0	0.0	0	1.2	9.7
$\Delta$ ssaJ	pre	4	0	0.0	0	1.0	0.0
	post (1-28)		0	2.0	2.7	1.1	13.5
$\Delta$ ssaT	pre	6	0	1.0	0	1.3	0.0
	post (1-28)		0	0.3	0	1.2	22.4

**Table 5.** Reduction in clinical signs in vaccinates post-challenge showing the efficacy of *S. dublin* vaccines.

Vaccine/ Strain	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
None	6	50.0	13.9	16.4	1.8	78.5
wild-type	5	0	0.0	0.0	1.2	39.5
$\Delta$ ssaC	6	0	0.0	0.0	1.0	39.3
$\Delta$ ssaJ	4	0	0.0	0.0	1.1	19.6
$\Delta$ ssaT	6	0	2.1	0.0	1.2	29.2

**E. Efficacy of a *S. typhimurium* *AssaC* or a *S. typhimurium* *AssaJ* or a *S. typhimurium* *AssaT* mutant as vaccines in cattle**

(2051-7923-I-MJK-98-006)

The safety and efficacy of a live attenuated *S. typhimurium* *AssaC* or *S. typhimurium* *AssaJ* or *S. typhimurium* *AssaT* mutant as vaccines was determined in cattle as described above in Example 2D. The results, shown in Tables 6 and 7 below, demonstrated that oral administration of each of these three mutants as a vaccine was safe and efficacious against experimentally induced salmonellosis. Protective effects seen with these SPI2 mutants were better than those observed with  $\Delta$ rfaK mutants.

**Table 6.** Lack of clinical signs following vaccination showing the safety of *S. typhimurium* vaccines.

Vaccine /Strain	Time	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
None	pre post (1-28)	6	0 0	0.0 0.4	0 0	1.0 1.4	0.0 7.6
<i>AssaC</i>	pre post (1-28)	6	0 0	0.0 0.3	0 0	1.0 1.3	0.0 84.8
<i>AssaJ</i>	pre post (1-28)	6	0 0	0.0 0.0	0 0	1.3 1.4	0.0 57.6
<i>AssaT</i>	pre post (1-28)	6	0 0	0.0 0.0	0 0	1.0 1.4	0.0 62.9
wild-type	pre post (1-28)	6	0 0	1.0 0.0	0 0	1.0 1.6	0.0 58.8

**Table 7.** Reduction in clinical signs in vaccinates post-challenge showing the efficacy of *S. typhimurium* vaccines.

Vaccine/ Strain	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
None	6	100.0	50.5	73.9	3.0	100

Vaccine/ Strain	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
<i>ΔssaC</i>	6	33.3	21.9	18.8	2.6	82.1
<i>ΔssaJ</i>	6	83.3	38.6	43.1	2.8	94.0
<i>ΔssaT</i>	6	50.0	33.0	39.5	2.4	81.0
wild-type	6	66.7	51.8	61.0	3.1	91.7

5 **F. Efficacy of a *S. dublin* *ΔssaC* or a *S. dublin* *ΔssaC/ΔssaT* or a *S. dublin* *ΔssaJ/ΔssaT* mutant or a combination of *S. dublin* *ΔssaC/ΔssaT* with *S. typhimurium* *ΔssaC/ΔssaT* as vaccines in cattle**

(2051-7923-I-MJK-99-001)

10 The safety and efficacy of a live attenuated *S. dublin* *ΔssaC* or *S. dublin* *ΔssaC/ΔssaT* or *S. dublin* *ΔssaJ/ΔssaT* mutant or a combination of *S. dublin* *ΔssaC/ΔssaT* with *S. typhimurium* *ΔssaC/ΔssaT* as vaccines was determined in cattle as described above in Example 2D. The results, shown in Tables 8 and 9 below, demonstrated that oral administration of each of these three mutants as a vaccine was safe and efficacious against experimentally induced salmonellosis. Results also

15 showed that there was no interference when a combination of *S. dublin* and *S. typhimurium* mutants were administered. Animals vaccinated with both *S. dublin* *ΔssaC/ΔssaT* and *S. typhimurium* *ΔssaC/ΔssaT* vaccine constructs had no mortality and the lowest clinical scores of all groups following challenge-exposure with a highly virulent heterologous *S. dublin* challenge strain. Because only partial

20 protection to challenge with *S. dublin* is afforded by vaccination of calves with live attenuated *S. typhimurium* vaccines, the resistance of these animals was likely due to immunity induced by the *S. dublin* component of the vaccine. Thus, efficacy of the *S. dublin* component of the vaccine was not interfered with by the presence of the *S. typhimurium* component, and it is possible that the latter may actually enhance

25 immunity to *S. dublin* when given as a combination vaccine.

**Table 8.** Lack of clinical signs following vaccination showing the safety of *S. dublin* single and double-deletion vaccines, and *S. dublin*/*S. typhimurium* combination vaccines.

	Vaccine	Time	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
5	None	pre	6	0	0	0	7.5	0
		post (1-28)		16.7	2.65	3.3	17.5	0
10	<i>S. dublin</i> <i>ΔssaC/T</i> <i>S. typhim.</i> <i>ΔssaC/T</i>	pre	6	0	0	0	7.5	0
		post (1-28)		0	0.89	0	17.5	0
15	<i>S. dublin</i> <i>ΔssaC</i>	pre	6	0	0	0	10.0	0
		post (1-28)		0	0.15	0	12.5	8.0
	<i>S. dublin</i> <i>ΔssaC/T</i>	pre	6	0	0	0	0	0
		post (1-28)		0	0.89	0	10.0	13.9
	<i>S. dublin</i> <i>ΔssaJ/T</i>	pre	6	0	1.0	0	1.4	8.3
		post (1-28)		0	1.03	0	1.4	26.8

**Table 9.** Reduction in clinical signs in vaccinates post-challenge showing the efficacy of *S. dublin* single and double-deletion vaccines, and *S. dublin*/*S. typhimurium* combination vaccines.

	Vaccine	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
20	None	6	100.0	36.1	33.2	50.0	100.0
	<i>S. dublin</i> <i>ΔssaC/T</i> <i>S. typhim</i> <i>ΔssaC/T.</i>	6	0	10.0	2.8	27.5	68.6
25	<i>S. dublin</i> <i>ΔssaC</i>	6	16.7	12.4	11.6	22.5	75.0
	<i>S. dublin</i> <i>ΔssaC/T</i>	6	20.0	12.7	14.8	20.0	78.6
30	<i>S. dublin</i> <i>ΔssaJ/T</i>	6	16.7	17.6	23.7	27.5	83.3

**G. Efficacy of a *S. typhimurium*  $\Delta$ ssaC or a *S. typhimurium*  $\Delta$ ssaC/ $\Delta$ ssaT or a *S. typhimurium*  $\Delta$ ssaJ/ $\Delta$ ssaT mutant or a combination of *S. dublin*  $\Delta$ ssaC/ $\Delta$ ssaT with *S. typhimurium*  $\Delta$ ssaC/ $\Delta$ ssaT as vaccines in cattle**  
(2051-7923-I-MJK-99-007)

5 The safety and efficacy of a live attenuated *S. typhimurium*  $\Delta$ ssaC or *S. typhimurium*  $\Delta$ ssaC/ $\Delta$ ssaT or *S. typhimurium*  $\Delta$ ssaJ/ $\Delta$ ssaT mutant or a combination of *S. dublin*  $\Delta$ ssaC/ $\Delta$ ssaT with *S. typhimurium*  $\Delta$ ssaC/ $\Delta$ ssaT as vaccines was determined in cattle challenged with virulent *S. typhimurium* as described above in Example 2D. The results, shown in Tables 10 and 11 below, demonstrated that oral  
10 administration of each of these three mutants as a vaccine was safe and efficacious against experimentally induced salmonellosis. As described above in Example 2F, these results also showed that there was no interference when a combination of *S. dublin* and *S. typhimurium* were administered.

15 **Table 10.** Lack of clinical signs following vaccination showing the safety of *S. typhimurium* vaccines.

Vaccine	Time	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
None	pre post (1-28)	6	0 0	0 0.45	4.2 0		0 0
<i>S. dublin</i> $\Delta$ ssaC/T <i>S. typhim</i> $\Delta$ ssaC/T.	pre post (1-28)	6	0 0	0 0	0 0		0 61.4
<i>S. typhim</i> $\Delta$ ssaC	pre post (1-28)	6	0 0	0 0	8.3 0.6		0 71.4
<i>S. typhim</i> $\Delta$ ssaC/T	pre post (1-28)	6	0 0	0 0	4.2 0		0 76.2
<i>S. typhim</i> $\Delta$ ssaJ/T	pre post (1-28)	6	0 0	0 0.74	4.2 0		0 71.2

**Table 11.** Reduction in clinical signs in vaccinates post-challenge showing the efficacy of *S. typhimurium* vaccines.

Vaccine	N =	Mortality (%)	Physical Condition	% Inactive Days	Fecal Score	% Shedding Days
None	6	100.0	36.98	32.6	77.5	100.0
<i>S. dublin</i> $\Delta$ ssaC/T <i>S. typhim</i> $\Delta$ ssaC/T.	6	16.7	11.11	1.4	25.0	82.2
<i>S. typhim</i> $\Delta$ ssaC	6	33.3	11.61	7.1	30.0	92.4
<i>S. typhim</i> $\Delta$ ssaC/T	6	0	9.23	5.6	22.5	86.9
<i>S. typhim</i> $\Delta$ ssaJ/T	6	16.7	18.4	18.4	30.0	79.0

Numerous modifications and variations of the above-described invention are expected to occur to those of skill in the art. Accordingly, only such limitations as appear in the appended claims should be placed thereon.